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(54) (-)-STRAND RNA VIRUS VECTOR HAVING AUTONOMOUSLY REPLICATING ACTIVITY

(57) A process for reconstituting virions of Sendai virus by introducing Sendai virus into a host in which early replication genes have been all expressed. This process makes it possible to produce a (-)-strand RNA virus vector with a high practical value

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Description

Field of the Invention

The present invention relates to a viral vector for the gene therapy. More specifically, this invention relates to a negative strand RNA viral vector.

Background of the Invention

As to the gene therapy for humans and animals, therapeutic effectiveness and safety are very important factors. Especially, therapy performed by using "viral vector" obtained by the viral gene recombination needs to be very cautiously carried out, when such undeniable possibilities exist as that gene may be inserted to unspecified sites of chromosomal DNA, that the recombinant virus and pathogenic virus may be released to the natural environment, and that the expression level of gene transfected into cells cannot be controlled, or the like, even though its therapeutic effectiveness is recognized.

These days, a great number of gene therapies using recombinant viruses are performed, and many clinical protocols of gene therapy are proposed. Characteristics of these recombinant viral vectors largely depend on those of viruses from which said vectors are derived.

The basic principle of viral vector is a method for transferring the desired gene into targeted cells by utilizing the viral infectivity. By "infectivity" in this specification is meant the "capability of a virus to transfer its nucleic acid, etc. into cells through its retaining adhesiveness to cells and fusion capability to membrane". With the surface of recombinant viral vectors genetically manipulated to insert a desired gene are associated the nucleocapsid and envelope proteins, etc. which are derived from the virus and confer the infectivity on the recombinant virus. These proteins enable the transfer of the enclosed recombinant gene into cells. Such recombinant viral vectors can be used for the purpose of not only gene therapy, but also production of cells expressing a desired gene as well as transgenic animals.

Viral vectors are classified into three classes comprising the retroviral vector, DNA viral vector and RNA viral vector.

These days, the vectors most frequently used in gene therapy are retroviral vectors derived from retroviruses. Retroviruses replicate through the following processes. First, upon viral infection established, they generate complementary DNAs (cDNAs) using their own reverse transcriptase as at least part of catalysts and their own RNA templates. After several steps, said cDNAs are incorporated into host chromosomal DNAs, becoming the proviruses. Proviruses are transcribed by the DNA-dependent RNA polymerase derived from the host, generating viral RNAs, which is packaged by the gene products encoded by their own genes, becoming viral particles.

In general, retroviral vectors used in gene therapy, etc. are capable of carrying out processes up to provirus generation. However, they are deficient viruses deprived of genes necessary for their packaging so that they do not form viral particles from provirus. Retroviruses are exemplified by, for example, mouse leukemia virus, feline leukemia virus, baboon type C oncovirus, human immunodeficiency virus, adult T cell leukemia virus, etc. Furthermore, recombinant retroviral vectors hitherto reported include those derived from mouse leukemia virus [see Virology, 65, 1202 (1991), Biotechniques, 9, 980 (1989), Nucleic Acids Research, 18, 3587 (1990), Molecular and Cellular Biology, 7, 887 (1987), Proceedings of National Academy of Sciences of United States of America, 90, 3539 (1993), Proceedings of National Academy of Sciences of United States of America, 86, 3519 (1989), etc.] and those derived from human immunodeficiency virus [see Journal of Clinical Investigation, 88, 1043 (1991)], etc.

Retroviral vectors are produced aiming at efficiently integrating a specific DNA into chromosomal DNA. However, since the insertion position of the desired gene is unpredictable, there is undeniable possibilities such as the damage of normal genes, activation of oncogenes, and excessive or suppressive expression of desired gene, due to inactivation by insertion. In order to solve these problems, a transient expression system using DNA viral vectors which can be used as extrachromosomal genes has been developed.

DNA viral vectors are derived from DNA viruses, having DNA as genetic information within viral particles. Replication of said DNA is carried out by repeating the process of generating complementary DNA strand using DNA-dependent DNA replicase derived from host as at least one of catalysts with its own DNA as template. The actual gene therapy using adenoviral vector, a DNA viral vector usable as extrachromosomal gene, is exemplified by the article in [Nature Genetics, 3, 1-2 (1993)]. However, since, in the case of DNA viral vectors, the occurrence of their undesirable recombination with chromosomal DNA within nucleus is also highly possible, they should be very carefully applied as vectors for gene therapy.

Recently, RNA viral vectors based on RNA viruses have been developed as conceivably more safer vectors than DNA viral vectors described above. RNA viruses replicate by repeating the processes for generating complementary strands using their own RNA-dependent RNA replicase as the catalyst with their own RNA as template.

The genome RNA of positive strand RNA viruses have dual functions as the messenger RNA (hereafter simply called mRNA), which generate proteins, depending on the translational functions of host cells, necessary for the repli-

cation and viral particle formation. In other words, the genome RNA itself of positive strand RNA viruses has a disseminative capability. In the present specification, by "disseminative capability" is meant "the capability to form infectious particles or their equivalent complexes and disseminate them to other cells following the transfer of nucleic acid into host cells by infection or artificial techniques and the intracellular replication of said nucleic acid". Sindbis virus classified to positive strand RNA viruses and Sendai virus classified to negative strand RNA viruses have both infectivity and disseminative capability. Adeno-satellite virus classified in Parbcviruses is infectious but not disseminative (mixed infection with adenovirus is required for the formation of viral particles). Furthermore, the positive strand RNA derived from Sindbis virus which is artificially transcribed *in vitro* is disseminative (forming infectious viral particles when transfected into cells), but neither positive nor negative RNA strands of Sendai virus artificially transcribed *in vitro* is disseminative (generating no infectious viral particles when transfected into cells).

In view of the advantage that the genome RNA functions as mRNA at the same time, the development of RNA viral vectors derived from positive strand RNA viruses preceded [see Bio/Technology, 11, 916-920 (1993), Nucleic Acids Research, 23, 1495-1501 (1995), Human Gene Therapy, 6, 1161-1167 (1995), Methods in Cell Biology, 43, 43-53 (1994), Methods in Cell Biology, 43, 55-78 (1994)]. For example, RNA viral vectors derived from Semliki forest virus (SFV) and Sindbis virus are basically of the RNA structure wherein the structural gene regions related to the viral structure are deleted, and a group of genes encoding proteins necessary for viral transcription and replication are retained with a desired foreign gene being linked downstream of the transcription promotor. Direct transfer of such recombinant RNA or cDNA which can transcribe said RNA [Nucleic Acids Research, 23, 1495-1501 (1995)] into cells by microinjection, etc. allows autonomous replication of RNA vectors containing the foreign gene, and the transcription of the foreign gene inserted downstream of the transcription promotor, resulting in the expression of the desired products from the foreign gene within cells. Furthermore, the present inventors succeeded in forming an infectious but not disseminative complex by the co-presence of cDNA unit (helper) for expressing the viral structural gene and that for expressing said RNA vector in the packaging cells. However, recombination between RNA derived from helper and vector RNA often occurred during packaging, resulting in the emergence of infectious particles. Then, it was elucidated that spike proteins present in the icosohedral capsid characteristic of positive strand RNA viruses catalyzed this recombination. Therefore, the introduction of variation into spike proteins has been attempted to solve these problems [Bio/Technology, 11, 916-920 (1993)].

Positive strand RNA viral vectors are expected to be useful as RNA vectors with autonomous replicating capability, but their use as vectors for gene therapy poses the following problems.

1. Since they are of the icosohedral structure, the size of foreign gene allowed to be inserted is limited to 3,700 nucleotides at most.

2. Until nucleic acids are released from the packaged complex into the cell and replicated, as many as five processes are required, including cellular adhesion, endocytosis, membrane fusion, decapsidation and translation of replication enzymes.

3. A possible formation of disseminative viral particles even in a minute quantity during packaging cannot be denied. Especially, even with attenuated viral particles, the inside RNA itself has disseminative potency and may belatedly be amplified, making it difficult to check.

4. Since these vectors are derived from viruses transmitted to animals by insects such as mosquitoes, when animals and humans to which such vector genes are transferred are mix-infected with wild type viruses, disseminative recombinants may be formed, possibly further creating a risk of said recombinants being scattered to the natural environment by insects.

Such problems described above are conceived to be basically overcome if RNA viral vectors derived from negative strand RNA viruses are constructed. That is, since negative strand RNA viruses do not have the capsid of icosohedral structure, and also since the envelope size of particles is known to vary depending on the inside RNA content, they are supposed to be much less restricted by the size of foreign genes to be inserted when used as RNA viral vectors. Furthermore, since a group of proteins required for transcription and replication are packaged into particles, only two processes are required, including cellular adhesion and membrane fusion, until nucleic acids are released from packaged complex and replicated. In addition, viral RNA alone is not disseminative, and disseminative particles can be easily identified, because they readily fuse with cell membrane and proliferate within cells. Therefore, the presence of disseminative particles can be easily detected. Furthermore, negative strand RNA viruses are not transmitted by insects.

In spite of many advantages of negative strand RNA viruses which may be used as the source of industrially useful viral vectors, no negative strand RNA vectors applicable for gene therapy has become available until now. This is probably due to tremendous difficulties in reconstituting viral particles via viral cDNA. Since the gene manipulation on the DNA level is required to insert foreign genes into vectors, so far as viral particles are not reconstructed from viral cDNA with a foreign gene inserted, it is difficult to use negative strand RNA viruses as a vector. "Reconstruction of viral particles" refers to the formation of original virus or recombinant virus *in vitro* or intracellularly from artificially prepared viral

genome nucleic acids

As described above, it has been clearly demonstrated that, even if RNA of negative strand RNA viruses (vRNA, viral RNA) or its complementary strand RNA (cRNA, complementary RNA) alone is transferred into cells, no negative strand RNA virus can be generated. This is a definitely different point from the case of positive strand RNA viruses. Although, in Tokkai H4-211377, "methods for preparing cDNA corresponding to negative strand RNA viral genome and infectious negative strand RNA virus" are described, the entire experiments of said document described in "EMBO J., 9, 379-384 (1990)" were later proved to be not reproducible, so that the authors themselves had to withdraw all the article contents [ref. EMBO J., 10, 3558 (1991)]. Therefore, it is obvious that techniques described in Tokkai H4-211377 do not correspond to the related art of the present invention.

With regard to the reconstitution system for negative strand RNA viruses, there are reports on influenza virus [Annu. Rev. Microbiol., 47, 765-790 (1993); Curr. Opin. Genet. Dev., 2, 77-81 (1992)]. Influenza virus is an eight-segmented negative strand RNA virus. According to these literatures, a foreign gene was first inserted to a cDNA corresponding to one of said segments, and the RNA transcribed from the cDNA corresponding to all eight segments including the one inserted with said foreign gene was assembled with the virus-derived NP protein to form an RNP. Then, the virus-reconstitution system was established by providing cells with these RNPs and RNA-dependent RNA polymerase. In addition, as with negative single stranded RNA viruses, virus-reconstitution from cDNA was reported with rabies virus belonging to rhabdoviruses [J. Virol., 68, 713-719 (1994)].

However, it has been difficult to use these virus reconstitution techniques as such for constructing vectors for gene therapy because of the following problems.

1. Reconstituted viruses were identified only by the expression of marker gene, RT-PCR, etc. No re-constitution system usable for the production of vector viruses in a satisfactory yield has been established.
2. Differing from the case of positive strand RNA viruses, in order to form complexes with infectivity but deficient in disseminative potency as vectors for gene therapy, it is necessary to enclose factors required for primary transcription and replication within the complex. No technique for amplifying these complexes in a large scale has been established.
3. For the purpose of intracellularly providing factors necessary for viral reconstitution, cells to which cDNAs are introduced are mix-infected with helper viruses such as wild type viruses and recombinant vaccinia virus, etc. It is not easy to separate these natural type viruses added.

Furthermore, as one problem with regard to RNA viral vectors in general, it is conceivably necessary to beforehand provide inhibitors for replication of RNA viral vectors which have no effects on host's replication and transcription, providing for the case where RNA replicated and transcribed in large amounts exerts undesirable effects on treated humans and animals. However, no such inhibitors have been developed.

Disclosure of the Invention

Problems to be solved by the present invention are to develop negative strand RNA viral vectors for practical use, methods for efficiently preparing said vectors, and inhibitors for the replication of said vectors.

Present inventors first attempted to reconstitute Sendai virus from nucleic acids of said virus which is a typical negative strand RNA virus, and conceived to be industrially most useful as a vector from the viewpoints of safety and convenience. First, in order to apply to the reconstitution test, various investigations were performed using cDNA derived from Sendai virus DI (defective interfering) particles [see EMBO J., 10, 3079-3085 (1991)] or cDNA of Sendai virus minigenome as experimental materials. As a result, they found efficient conditions regarding weight ratios among materials to be transferred into host cells, including cDNA, cDNAs concerning the transcription and replication, and the recombinant vaccinia virus to provide a unit for expressing the T7RNA polymerase. Furthermore, the present inventors obtained full-length cDNAs corresponding to both positive and negative strands, constructed plasmids for inducing the intracellular biosynthesis of either positive or negative strand RNA of Sendai virus, and transferred said plasmids into host cells wherein cDNAs concerning the transcription and replication were expressed. As a result, they first succeeded in re-constructing Sendai virus particles from cDNAs derived thereof.

In addition, the present inventors found that Sendai virus could be reconstructed without using recombinant vaccinia virus as T7-RNA polymerase expression unit. That is, when the full-length RNA of Sendai virus transcribed *in vitro* was transferred into cells, and cDNAs encoding enzymes for initial transcription and replication were transcribed under the control of T7 promotor, viral particles were re-constructed. This indicates that, if cells which express group of all enzymes required for initial transcription and replication are constituted, the recombinant Sendai virus, eventually complexes described above can be formed entirely without using helper viruses such as vaccinia virus. Since cells which express group of all enzymes required for initial transcription and replication were already described [J. Virology, 68, 8413-8417 (1994)], those skilled in the art may form such cells with reference to said article. Cells described in said ref-

erence are the one derived from the 293 cell line which carries three of Sendai virus genes, namely NP, P/C and L, on its chromosome, expressing proteins encoded by the three genes, NP, P/C and L.

From numerous examples of viral vectors, if viral particles can be efficiently reconstructed from nucleic acids, it is obvious that those skilled in the art are able to readily exchange a desired viral gene, insert a foreign gene, or inactivate or delete a desired gene. For example, an article on the use of DI particles [J. Virol., 68, 8413-8417 (1994)] clearly indicates that, when RNA deficient in at least a part of structural genes of Sendai virus, but normal of genes for replication enzymes is transferred into cells, the succeeding autonomous replication may be able to proceed, if group of enzymes necessary for the initial transcription and replication are provided in the cells. Therefore, once an RNA molecule containing a foreign gene transcribed from "specific viral cDNA deficient in at least a part of structural genes but normal in genes for the replication enzyme group" can be enclosed in the viral structure comprising the initial transcription and replication enzymes, complexes which are infectious to and autonomously replicating but deficient in the disseminative potency, and functional as the foreign gene vector can be formed. Such complexes are extremely useful as a vector for gene therapy. That is, in the present invention, with a negative strand RNA virus, it becomes possible to prepare complexes which are infectious as well as autonomously replicative but is deficient in the disseminative potency, for example, complexes comprising the initial transcription and replication enzymes.

The present inventors further developed a method for amplifying the same complex by transfecting said complex to cells which express the structural proteins corresponding to genes in RNA of the complex which have been deleted or inactivated. Further, taking avian eggs into consideration as the most suitable medium for proliferating Sendai virus in order to amplify the said complex, the inventors found that transgenic avians, their eggs and cells which carry at least one or more genes out of M, F and HN genes of Sendai virus on chromosome are suitable for amplifying complexes. Methods for preparing transgenic avians have been reported [Poultry Sci., 65, 1445-1458 (1986); Bio/Technology, 12, 60-63 (1994)], and those skilled in the art can appropriately produce transgenic birds carrying at least one or more genes out of M, F and HN genes on their chromosomes. Preferably, proteins encoded by genes related to the deficiency in disseminative capability of RNA contained in the complex among M, F and HN genes,are expressed in transgenic birds.

The present inventors also developed a method for preparing the complex described above. In the following, cases related to Sendai virus are exemplified. Genome of Sendai virus Z is a single stranded RNA comprising 15384 nucleotides [Virology, 108, 318-324 (1981)]. Its entire base sequence has been determined from cDNA clones prepared by using reverse transcriptase [Nucleic Acids Research, 11, 7313-7330 (1983); Nucleic Acids Research, 12, 7965-7972 (1984); Nucleic Acids Research, 14, 1545-1563 (1986)]. Since its genome RNA is a negative strand, a group of enzymes for transcription and replication in the viral particles perform both transcription and replication with the genome RNA as template. At least six proteins including NP, P/C, M, F, HN and L are known as proteins encoded by the genome RNA. It has been elucidated that, of these proteins, NP, P/C and L are factors essential and sufficient for replication [Journal of Virology, 68, 8413-8417 (1994)], and M, F and HN are components necessary for constructing the viral structure. Based on these facts, when a specific RNA virus from which RNA is derived is Sendai virus, it is possible to reconstruct an infectious complex by transferring both 1) cDNA transcribable to DNA, and 2) a gene encoding the RNA polymerase necessary for transcribing said cDNA within cells or an RNA molecule itself transcribed from said cDNA *in vitro* into cells wherein all the genes for the autonomous replication, NP, P/C and L, and a group of genes, out of M, F and HN genes, for the deficiency of RNA dissemination are expressed. In this case, all genes for the autonomous replication, NP, P/C and L, and genes, out of M, F and HN genes, for the deficiency of RNA disseminative capability may be transiently expressed by transfecting cells with the plasmids coding for the respective genes. However,genes related to the deficiency of RNA disseminative capability at least are preferably incorporated into chromosomes to be stably expressed.

The present inventors further developed a method for producing said complex thus re-constituted in large quantities, wherein said complex is replicated by transfecting it to cells having no genes related to the autonomous replication but expressing genes, from among M, F and HN genes, related to the deficiency in the RNA disseminative potency. In this case, as cells having no genes related to the autonomous replication but expressing a group of genes, from among M, F and HN genes, related to the deficiency in the RNA disseminative capability, transgenic avian eggs expressing said group of genes are preferable for the production of complex on a large scale.

Furthermore, the present inventors produced cells for propagating the complex containing said RNA and proteins. More specifically, said cells are those with genes corresponding to a group of genes related to the deficiency in infectious particle-forming capability of the RNA retained by said complex, and capable of intracellularly producing proteins encoded by said genes. In the case wherein the specific RNA virus from which RNA is derived is Sendai virus, cells which have at least more than one genes from among M, F and HN genes on their chromosomes or animals having such cells are used. In addition, M, F and HN genes are not necessarily of wild type. Any of those with functions equivalent to those of the wild type will be usable. That is, any gene may be used where said gene has complementarity to the wild type for deficient virus when functionally introduced into cells. Preferable cells to be used are host cells for Sendai virus. It is preferable that proteins encoded by genes corresponding to those related to the deficiency in infec-

tious particle-forming capability, from among M, F and HN genes in the vector viral RNA, are intracellularly produced.

Hitherto only the enhancement of expression efficiency has been emphasized with conventional RNA virus vectors, and little efforts have been made for developing compounds to suppress the RNA replication to prevent unfavorable results due to excessive expression. In this respect, the present inventors developed an inhibitor for the negative strand virus vector which specifically inhibits the RNA-dependent RNA replication and RNA-dependent RNA transcription without affecting the transcription and translation of cell-derived RNAs leading only to the inhibition of RNA-dependent RNA replication.

That is, the present invention comprises the followings.

1. A complex comprising an RNA molecule derived from a specific disseminative negative strand RNA virus and viral structural components containing no nucleic acids, having the infectivity and autonomous RNA replicating capability, but deficient in the disseminative capability.

2. The complex of description 1, wherein said specific RNA virus is a negative strand RNA virus having non-segmented genome.

3. The complex of description 2, wherein said specific RNA virus is Sendai virus.

4. An RNA molecule comprising Sendai viral RNA or Sendai viral cRNA, wherein said RNA molecule is defective in that at least more than one gene coding for the M, F and HN proteins are deleted or inactivated.

5. A complex comprising the RNA of description 4 and viral structural components containing no nucleic acids derived from Sendai virus, having the infectivity and autonomous RNA replicating capability, but deficient in the disseminative capability.

6. A DNA molecule comprising a template DNA transcribable to the RNA molecule of description 4 *in vitro* or intracellularly.

7. The complex of any one of descriptions 1-3 or 5, wherein the RNA molecule contained in said complex comprises a foreign gene.

8. The complex of descriptions 3 or 5, wherein the RNA molecule contained in said complex comprises a foreign gene.

9. The RNA molecule of description 4 comprising a foreign gene.

10. The DNA molecule of description 6 comprising a foreign gene.

11. An inhibitor for RNA replication contained in the complex of any one of descriptions 1-3, 5, 7 or 8 comprising an inhibitory drug for the RNA-dependent RNA replication.

12. A host whereto the complex of any one of descriptions 1-3, 5, 7 or 8 can disseminate.

13. The host of description 12 comprising a group of genes related to the infectivity of the complex of any one of descriptions 1-3, 5, 7 or 8 on its chromosomes, and capable of replicating the same copies of said complex when infected with it.

14. The host of descriptions 12 or 13, wherein said host is animals, or cells, tissues, or eggs derived from it.

15. The host of description 14 wherein said animal is mammalian.

16. The host of description 14 wherein said animal is avian.

17. A host comprising a group of genes related to the infectivity of the complex of any one of descriptions 3, 5 or 8 on its chromosomes, and capable of replicating the same copies of said complex when infected with it.

18. A host comprising at least more than one gene of the M, F and HN genes of Sendai virus or genes having functions equivalent to them on its chromosomes.

19. A host comprising the M gene of Sendai virus or its functionally equivalent gene on its chromosomes.

20. A host comprising the M, NP, P/C and L genes of Sendai virus on its chromosomes (wherein each gene may be substituted with its functionally equivalent gene, respectively).

21. A host comprising the M, F and HN genes of Sendai virus on its chromosomes (wherein each gene may be substituted with its functionally equivalent gene, respectively).

22. A host comprising the M, F, HN, NP, P/C and L genes of Sendai virus on its chromosomes (wherein each gene may be substituted with its functionally equivalent gene, respectively).

23. The host of any one of descriptions 17-22, wherein said host is animal, or cell, tissue or egg derived from it.

24. The host of description 23, wherein said animal is mammalian.

25. The host of description 23, wherein said animal is avian.

26. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of descriptions 1-3, 5, 7 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,

b. a group of enzymes required for replicating said RNA or said cRNA, or a unit capable of biosynthesizing said group of enzymes, and

c. a group of proteins related to the infectivity of said complex, or a unit for biosynthesizing said group of pro-

teins.

27. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of descriptions 1-3, 5, 7 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. a group of enzymes required for replicating said RNA or said cRNA, or a unit capable of biosynthesizing said group of enzymes, and
c. the host of any one of descriptions 12-25.

28. A kit consisting of the following two components,

a. the complex of any one of descriptions 1-3, 5, 7 or 8, and
b. the host of any one of descriptions 12-25.

29. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of descriptions 3, 5 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. all NP, P/C and L proteins of Sendai virus, or a unit for biosynthesizing said group of proteins, and
c. a group of proteins related to the infectivity of said complex, or a unit for biosynthesizing said group of proteins.

30. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of descriptions 3, 5 or 8, cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. all NP, P/C and L proteins of Sendai virus, or a unit capable of biosynthesizing said group of proteins, and
c. the host of any one of descriptions 17-25.

31. A kit consisting of the following two components,

a. the complex of any one of descriptions 3, 5 or 8, and
b. the host of any one of descriptions 17-25.

32. A method for producing the complex of any one of descriptions 1-3, 5, 7 or 8 by introducing three components of descriptions 26a, 26b and 26c into a host.
33. A method for producing the complex of any one of descriptions 1-3, 5, 7 or 8 by introducing both components of descriptions 27a and 27b into the host of description 27c.
34. A method for amplifying and producing the complex of description 28a by transfecting said complex to the host of description 28b.
35. A method for producing the complex of any one of descriptions 3, 5 or 8 by introducing the three components of descriptions 29a, 29b and 29c into a host.
36. A method for producing the complex of any one of descriptions 3, 5 or 8 by introducing both components of descriptions 30a and 30b into the host of description 30c.
37. A method for amplifying and producing the complex of description 31a by transfecting said complex into the host of description 31b.
38. The RNA molecule of description 9 wherein a gene corresponding to the M gene is deleted or inactivated.
39. The RNA molecule of description 9 wherein all the genes corresponding to the M, F and HN genes are deleted or inactivated.
40. A kit consisting of the following three components,

a. the RNA molecule of description 38,
b. the host of description 20, and
c. the host of description 19.

41. A method for producing a complex by introducing the RNA molecule of description 40a into the host of description 40b, and amplifying and producing said complex by transfecting it into the host of description 40c.

42. A complex produced by the method of description 41.

43. A kit consisting of the following three components,

a. the RNA molecule of description 39,
b. the host of description 22, and
c. the host of description 21

44. A method for producing a complex by introducing the RNA molecule of description 43a into the host of description 43b, and amplifying and producing said complex by transfecting it into the host of description 43c

45. A complex produced by the method of description 44.

46. An inhibitor for RNA replication contained in the complex of either descriptions 42 or 45 comprising an inhibitory drug of the RNA-dependent RNA replication

47. A method for preparing the foreign proteins, wherein said method comprises the process of introducing the complex of description 7 to a host and the process of recovering the expressed foreign proteins.

48. A method for preparing the foreign proteins of description 47, wherein the host is a cell expressing a group of genes, from among those related to the disseminative capability, which are deficient in the RNA molecule contained in the complex of description 7.

49. A culture medium or chorio-allantoic fluid containing the expressed foreign proteins, wherein said culture medium or chorio-allantoic fluid is obtained by inoculating the complex of description 7 into a host and recovering it.

Any negative strand RNA viruses with disseminative capability may be used as materials in the present invention. Although incomplete viruses such as defective interfering particles (DI particles) and synthetic oligonucleotide may also be used as partial materials, in general, they must have the base sequence equivalent to that of the virus with disseminative capability. Negative strand RNA viruses of the present invention include, for example, Sendai virus, Newcastle disease virus, mumps virus, measles virus, respiratory syncytial virus, rinderpest virus of cattle and canine distemper virus of Paramyxoviridae, influenza virus of Orthomyxoviridae, vesicular stomatitis virus and rabies virus of Rhabdoviridae.

As the negative strand viral material, recombinant negative strand viruses derived from any viruses described above and retaining the disseminative capability may be used. For example, the recombinant negative strand virus may be the one with the gene for the immunogenicity inactivated or a partial region of gene altered to enhance the efficiency of RNA transcription and replication.

RNAs contained in the RNA-protein complex of the present invention can be obtained by transcribing modified cDNAs derived from any viruses or recombinant viruses described above *in vitro* or intracellularly. In RNAs thus obtained, at least one gene related to the disseminative capability of the original virus must be deleted or inactivated, but the gene related to the autonomous replication should not. In addition, RNA molecules with artificial sequences, which are obtained by transcribing, *in vitro* or intracellularly, DNA formed by inserting the genes for the autonomous replication into cDNA having both terminus structures of the virus genome such as DI molecule, may be also used.

As described above, in the case of Sendai virus, "the genes related to autonomous replication" refer to any one of the NP, P/C and L genes, and "the gene related to the disseminative capability" refers to any one of the M, F and HN genes. Therefore, the RNA molecule of Sendai virus Z strain deficient only in the M gene, for example, is suitable as a component contained in the "complex" of the present invention. Also, the RNA molecule having all the M, F and HN genes deleted or inactivated are also preferable as the component contained in the "complex" of the present invention. On the other hand, it is necessary for the genes encoding the NP, P/C and L proteins to be expressed from RNA. However, the sequences of these genes are not necessarily the same as those of virus, and may be modified by introducing variations, or replacing by the corresponding gene derived from other viruses, so far as the transcription and replication activity of the resulting RNA is similar to or higher than that of the natural one.

"Virus structural component free of nucleic acid" of the present invention includes, for example, virus with only its RNA removed. As such structural component is used the one which complements the infectivity and autonomous replicating capability at the early stage, but not the disseminative capability. In the case of Sendai virus, the complex composed of its RNA with only the M gene deleted, and Sendai virus having only its RNA deleted have the infectivity and autonomous replicating capability, but no disseminative capability. Complex may contain other components so long as it is provided with no disseminative capability. For example, complex may contain adhering molecule, ligand, receptors, etc. on its envelope surface for facilitating the adherence to specific cells.

The RNA molecule contained in the complex can have an inserted foreign gene at its appropriate site. In order to express a desired protein, the foreign gene encoding said protein is inserted. In the case of Sendai viral RNA, a sequence of bases of 6 multiplication in number is preferably inserted between sequences R1 (5'-AGGGTCAAAGT-3') and R2 (5'-GTAAGAAAAA-3') [Journal of Virology, Vol. 67, No. 8 (1993), p.4822-4830]. Levels of expression of the foreign gene inserted into RNA can be regulated by virtue of the site of gene insertion and the base sequence flanking the

foreign gene. For example, in the case of Sendai viral RNA, it is known that there are increasing levels of expression of the inserted gene with decreasing distance of said gene from the NP gene. Preferred host cells for the introduction of the complex to express desired proteins are those expressing genes deleted in the RNA molecule composed of said complex. For this, transgenic avian eggs expressing said genes are most preferable for preparing proteins in large quantities. For example, proteins thus expressed can be recovered from the culture medium when host cells are cultured cells, and chorio-allantoic fluid when host cells are chicken eggs, using standard techniques. In Examples 5 and 6 is used a disseminative complex in place of non-disseminative complex of the present invention. However, it will be clear to those skilled in the art that similar results are obtained with the complex of the present invention as with the disseminative complex in these examples when "cells expressing genes deleted from among genes for disseminative capability in the RNA molecule contained in the complex" are used as host cells.

Furthermore, the present inventor has confirmed that, for the efficient reconstitution of Sendai virus particles, cDNA to be introduced into cells is preferably in the circular form rather than in the linear form, and, for viral particle formation at a high efficiency, the transcription of the positive strand RNA is preferred to that of the negative strand RNA within cells. Although these conditions may not necessarily be applicable to the reconstitution of all other negative strand RNA viruses, it is possible to search for appropriate conditions for the reconstitution of other negative strand RNA viruses based on the disclosure of the present invention and conventional technology, indicating a possibility for establishing techniques to produce basic materials of desired negative strand viral vectors, that is, the viral reconstitution systems.

As the "RNA replication inhibitor" of the present invention, any drugs to inhibit RNA-dependent RNA replication may be applied, and, for example, Ribavirin, TJ13025, etc. are preferably used. Such replication inhibitors are effective, for example, when health deterioration is noticed with the cellular amplification of recombinant RNA, or when the control of intracellular expression of foreign genes derived from recombinant RNA is required.

As an embodiment of the present invention, processes for reconstituting the complex of the present invention from cDNA with the M gene deleted of Sendai virus (steps A-B), and those for amplifying said complex (steps B-C) are shown in Fig. 1.

Brief description of the drawings

Figure 1 is a schematic representation of a process for generating complexes of the present invention from cDNA deficient in the M gene of Sendai virus (steps A→B) and further amplifying said complexes (steps B→C).

Figure 2 is a schematic representation of the construction of a pUC18/T7(+)HVJRz.DNA.

Figure 3 is a schematic representation of the construction of a pUC18/T7(-)HVJRz.DNA.

Figure 4 is a graphical representation showing the relationship between the time after the infection of SeVgp120 into CV-1 cells and levels of HAU and gp120 expression.

Best mode for carrying out the Invention

In the following, the present invention will be concretely described with reference to Examples, but not be limited to them.

Example 1. Preparation of Sendai virus transcription units pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA

Plasmid pUC18/T7(-)HVJRz.DNA was constructed by inserting a DNA molecule comprising T7 RNA polymerase promotor, Sendai virus cDNA designed to be transcribed to the negative strand RNA and the ribozyme gene in this order into pUC18 vector. Also, plasmid pUC18/T7(+)HVJRz.DNA was constructed by inserting a DNA molecule comprising T7 RNA polymerase promotor, Sendai virus cDNA designed to be transcribed to the positive strand RNA and the ribozyme gene in this order into pUC18 vector. Constructions of pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA are shown in Figs. 1 and 2, respectively.

Example 2. Reconstitution experiment of Sendai virus from cDNA

LLC-MK2 cells (2×10^6) trypsinized in a usual manner were placed in a 60-mm diameter plastic dish, and incubated in MEM medium (MEM supplemented with 10% FBS) (2 ml) in a 5% CO_2 atmosphere at 37°C for 24 h. After removing the medium and washing with PBS (1 ml), a suspension of recombinant vaccinia virus vTF7-3 expressing T7 polymerase in PBS (0.1 ml) was added to the cells at the multiplicity of infection (moi) of 2. The dish was gently agitated every 15 min to thoroughly spread the viral solution for 1 h infection. After removing the viral solution and washing with PBS (1 ml), a medium containing cDNA, which was prepared as follows, was added to the dish.

Nucleic acids shown in Tables 1 and 2 (containing plasmids expressing factors required for the replication of Sendai virus, pGEM-L, pGEM-P/C and pGEM-NP) were placed in a 1.5-ml sampling tube, and adjusted to a total volume of 0.1

ml with HBS (Hepes buffered saline: 20 mM Hepes pH 7.4 containing 150 mM NaCl) in these tables. (-) and (+)cDNAs represent plasmids pUC18/T7(-)HVJRz DNA and pUC18/T7(+)HVJRz DNA, respectively, and C and L indicate that cDNA is introduced into cells in the circular form and linear form after the treatment with restriction enzyme MluI, respectively.

On the other hand, in a polystyrene tube were placed HBS (0.07 ml), DOTAP (Boehringer Mannheim, 0.03 ml). To this tube was added the nucleic acid solution described above, and the mixture was left standing as such for 10 min. Then, to this mixture was added the cell culture medium described above (2 ml, MEM supplemented with 10% FBS) followed by the vaccinia virus inhibitors rifampicin and cytosine arabinoside C (C/Ara/C), to the final concentrations of 0.1 mg/ml and 0.04 mg/ml, respectively, resulting in the preparation of the medium containing cDNA described above.

The dish described above was incubated in a 5% CO_2 atmosphere at 37°C for 40 h. The cells in the dish were harvested using a rubber policeman, transferred to an Eppendorf tube, sedimented by centrifuging at 6,000 rpm for 5 min, and re-suspended in PBS (1 ml). Aliquots of this cell suspension, as such or after diluted, were inoculated to 10-days old developing embryonated chicken eggs. That is, the cell suspension was diluted with PBS to the cell numbers shown in Table 1, and eggs inoculated with its 0.5-ml aliquots were incubated at 35°C for 72 h, then at 4°C overnight. Chorioallantoic fluid was recovered as virus solution from these eggs using a syringe with a needle.

Hemagglutinin unit (HAU) and plaque forming unit (PFU) of the recovered virus solution were assayed as follows.

HAU was determined as follows. Chicken blood was centrifuged at 400 x g for 10 min and the supernatant was discarded. Precipitates thus obtained were suspended in 100 volumes of PBS, and centrifuged at 400 x g for 10 min to discard the supernatant. This procedure was repeated twice to prepare an 0.1% blood cell solution. Two-fold serial dilutions of virus solutions were prepared, and 0.05 ml each dilution to be assayed was dispensed into each well of 96-well titer plate. The blood cell solution (0.05 ml each) was further added to each well, gently swirled to ensure a thorough mixing, and left at 4°C for 40 min. The highest virus dilution to cause the hemagglutination observable with the naked eye was taken as HAU.

PFU was assayed as follows. CV-1 cells were grown to a monolayer on a 6-well culture plate. After the culture medium was discarded, a virus solution 10-fold serially diluted (0.1 ml each) was dispensed into each well of the culture plate to infect the cells at 37°C for 1 h. During the infection, a mixture of 2 x MEM free of serum and melted 2% agar (55°C) was prepared, and trypsin was added to the mixture to a final concentration of 0.0075 mg/ml. After 1 h infection and removal of the virus solution, the culture medium mixed with agar (3 ml each) was added to each well of the culture plate, and incubated under a 5% CO_2 atmosphere at 37°C for 3 days. Phenol red (0.1%) (0.2 ml) was added to each well, incubated at 37°C for 3 h, and then removed. Unstained plaques were counted to estimate the virus titer as PFU/ml.

Table 1 shows Sendai virus template cDNAs transfected into LLC-2 cells, amounts of cDNA factors, pGEM-L, pGEM-P/C, and pGEM-NP, required for the RNA replication, incubation time, cell numbers inoculated to chicken eggs, HAU and PFU values.

Table 1

Template cDNA	Total amount (μg)	pGEM -L (μg)	pGEM -P/C (μg)	pGEM -NP (μg)	Incubation time (h)	Amount of cells	HAU	PFU
(+)cDNA/C	10	4	2	4	40	1.00×10^5	512	2×10^9
(+)cDNA/C	10	4	2	4	40	1.00×10^5	256	9×10^8
(+)cDNA/C	10	4	2	4	40	1.00×10^6	256	9×10^8
(+)cDNA/L	10	4	2	4	40	1.00×10^5	<2	<10
(+)cDNA/L	10	4	2	4	40	1.00×10^5	<2	<10
(+)cDNA/L	10	4	2	4	40	1.00×10^6	<2	<10
(-)cDNA/L	10	4	2	4	40	1.00×10^4	<2	<10
(-)cDNA/L	10	4	2	4	40	1.00×10^5	<2	<10
(-)cDNA/L	10	4	2	4	40	1.00×10^6	<2	<10
(-)cDNA/C	10	4	2	4	40	1.00×10^4	<2	<10
(-)cDNA/C	10	4	2	4	40	1.00×10^5	<2	<10
(-)cDNA/C	10	4	2	4	40	1.00×10^5	4	8×10^3

Samples showing both HAU and PFU were sedimented by ultra-centrifugation, re-suspended, purified by a sucrose density gradient centrifugation from 20% to 60%, and fractionated by 12.5% SDS-PAGE. Each protein contained in these samples was the same in size as that of Sendai virus.

These results demonstrated that Sendai virus can be reconstituted by introducing cDNAs into cells, and that virus particles are more efficiently reconstituted by introducing cDNAs transcribing positive strand RNAs as compared with those transcribing negative strand RNAs, and further by introducing cDNAs in the circular form rather in the linear form.

Example 3. Survey of RNA replication factors required for Sendai virus reconstitution

Experiments were performed to examine whether all three plasmids expressing the L, P/C and NP proteins were required for the reconstitution of Sendai virus. Experimental methods were similar to those described in Example 2 except that any combinations of two out of pGEM-L, pGEM-P/C and pGEM-NP plasmids or only one out of them, instead of all these three combined as in Example 2, were introduced together with a template cDNA into cells.

Table 2 shows Sendai virus template cDNAs introduced into LLC-MK2 cells, amounts of the cDNA factors required for RNA replication including pGEM-L, pGEM-P/C and pGEM-NP, incubation time, number of cells inoculated into chicken eggs, and values of HAU and PFU.

Table 2

Template cDNA	Total amount (μg)	pGEM -L	pGEM -P/C	pGEM -NP	Incubation time (h)	Number of cells inocu-lated	HAU	PFU
(+)cDNA/C	10	4	2	4	40	$1.00x10^5$	256	$6x10^8$
(+)cDNA/C	10	4	2	4	40	$1.00x10^6$	512	$4x10^9$
(+)cDNA/C	10	0	2	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	0	2	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	0	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA	10	0	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	0	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/c	10	0	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	0	40	$1.00x10^6$	<2	<10

As shown in Table 2, no virus reconstitution was observed by introducing any combinations of two out of these three factors into cells, confirming the necessity of all three proteins L, P/C and NP for the virus reconstitution. Example 4. Reconstitution experiment of Sendai virus *in vitro* from transcribed RNAs

Since the reconstitution of Sendai virus from the functional cDNA clones was described in Example 2, it was further examined whether transcription products of said cDNAs *in vitro*, that is, vRNA and cRNA, can support similar reconstitution.

After the Sendai virus transcription units, pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA, were linearized with restriction enzyme M1uI, using these DNAs as templates, RNA synthesis was performed *in vitro* with a purified T7 polymerase preparation (EPICENTRE TECHNOLOGIES: Ampliscribe T7 Transcription Kit). The method for synthesizing in vitro RNAs essentially followed the protocols provided with the kit. Using RNA products thus obtained in place of cDNAs in Example 2, similar experiments were performed, and the virus production was estimated by HA test. Results are shown in Table 3.

Table 3

Template cDNA	Total amount (μg)	pGEM-L (μg)	pGEM-P/C (μg)	pGEM-NP (μg)	Incubation time (h)	Number of cells inoculated	HAU	PFU
in vitro (-)RNA	10	4	2	4	40	$1.00x10^6$	512	$2x10^9$
in vitro (-)RNA	10	4	2	4	40	$1.00x10^6$	512	ND
in vitro (+)RNA	10	4	2	4	40	$1.00x10^6$	2	$5x10^3$
in vitro (+)RNA	10	4	2	4	40	$1.00x10^6$	<2	ND

These results indicate that virus can be reconstituted by introducing either negative or positive sense strand RNAs into cells.

Example 5. Expression of foreign genes inserted into Sendai viral vectors in host cells

1. Preparation of Sendai virus vector "pSeVgp120" inserted with a foreign gene (HIV-1 gp120)

Using a set of primers comprising

primer a (5'-TGCGGCCGCCGTACGGTGGCAATGAGTGAAGGAGAAGT-3') (SEQ ID NO:1) and primer d (5'-TTGCGCCCGCGATGAACTTTCACCCTAAGTTTTTATTACTACGGCG-TACGTCATCTTTTTTCTCTCTGC-3') (SEQ ID NO:2),

the HIV-1gp120 gene was amplified on "pN1432" by the standard PCR techniques. PCR products were subjected to TA cloning, digested with NotI, and then inserted into the NotI site of "pSeV18⁺". Then, *E. coli* cells were transformed with this recombinant plasmid. DNAs were extracted from each colony of *E. coli* by the "Miniprep" method, digested with DraIII, and then electrophoresed. Positive clones (designated "clone 9" hereafter) were selected by confirming to contain DNA fragments of the size expected from the insertion. After DNA fragments were confirmed to have the authentic nucleotide sequence, DNAs were purified by a cesium chloride density gradient centrifugation. pSeV18⁺ inserted with the gp120 gene is designated "pSeVgp120" hereafter.

2. Reconstitution of Sendai virus containing pSeVgp120 (SeVgp120) and analysis of gp120 expression

Except for the further transfection of pSeVgp120 into LLCMK2 cells, in addition to pGEM-NP, pGEM-P/C and pGEM-L, chorio-allantoic fluid was recovered from embryonated chicken eggs and assayed for the viral HAU by exactly as described in Example 2. The recovered virus was also examined for the expression of gp120 by ELISA as follows.

Samples (100 μl each) were dispensed into each well of a 96-well plate which had been coated with monoclonal antibody against HIV-1, and incubated at 37°C for 60 min. After washing with PBS, HRP-linked anti-HIV-1 antibody (100 μl each) was added to each well, and incubated at 37°C for 60 min. After washing with PBS, tetramethylbenzidine was added to each well, and amounts of reaction product converted by the action of HRP under acidic conditions were determined by following the optical density at 450 nm to estimate the expression amount of gp120. Results are shown in the left-hand column in Table 4.

The virus solution thus obtained was inoculated to CV-1 cells, and similarly examined as follows. CV-1 cells were dispensed to a culture plate at 5 x 10^5 cells/plate, grown, and then the culture medium was discarded. After washing with PBS(-), the viral solution was added to the cells at the multiplicity of infection of 10, and incubated at room temper-

ature for 1 h. After the virus solution was discarded, washed with PBS(-), a plain MEM medium (MEM medium supplemented with antibiotics AraC and Rif. and trypsin) was added to the cells, and incubated at 37°C for 48 h. After the reaction, the medium was recovered and assayed for HAU (by a similar method as described in Example 2) and examined for the expression of gp120 (by ELISA). Results are shown in the center column of Table 4. In addition, the supernatant of CV-1 cell culture medium was inoculated to embryonated chicken eggs again, and the virus solution thus obtained was assayed for HAU and also examined for the gp120 expression (by ELISA). Results are shown in the right hand column of Table 4.

Table4

(μg/ml)

Chorio-allantoic fluid (F1) gp120 (HAU)	CV-1 medium (F1) gp120 (HAU)	Chorio-allantoic fluid (F2) gp120 (HAU)
0.10 (4)	3.46 (128)	
0.15 (32)	1.81 (128)	1.56, 1.21 (512, 512)
0.05 (32)	2.20 (128)	

As shown in Table 4, markedly high concentrations of gp120 were detected in CV-1 cells in culture (center column of the Table), and also in the chorio-allantoic fluids from embryonated chicken eggs inoculated again with the virus (right-hand column of the Table). In the left-hand and center columns of the Table are shown the mean values of three clones.

Furthermore, the expression of gp120 was analyzed by Western blotting. After the culture medium of CV-1 cells infected with SeVgp120 was centrifuged at 20,000 rpm for 1 h to sediment virus, the supernatant was treated with either TCA (10%, v/v) for 15 min on ice or 70% ethanol at -20°C, and centrifuged at 15,000 rpm for 15 min. Proteins thus precipitated were mixed to react with an "SDS-PAGE sample buffer" (Daiichi Chemicals) at 90°C for 3 min, and then subjected to electrophoresis on 10% SDS-polyacrylamide gel (SDS-PAGE). Proteins thus fractionated were transferred to PVDF membranes (Daiichi Chemicals), reacted with monoclonal antibody 902 at room temperature for 1 h, and then washed with T-TBS. The membranes were reacted with anti-mIgG (Amersham) at room temperature for 1 h, and washed with T-TBS. The membranes were then reacted with HRP-linked protein A (Amersham) at room temperature for 1 h, washed with T-TBS, and 4-chloro-1-naphthol (4CNPlus) (Daiichi Chemicals) was added to detect gp120. As a result, protein bands were visualized at positions corresponding to the expected molecular weight of gp120.

In addition, effects of postinfection time of CV-1 cells transfected with SeVgp120 on the HAU value and gp120 expression amount were analyzed. CV-1 cells (5 x 10^6) dispensed to 10-cm plate were infected with SeVgp120 at the multiplicity of infection of 10, and the culture medium (1 ml each) was postinfectionally recovered at 30, 43, 53 and 70 h, mixed with an equal volume of the fresh medium, and subjected to HAU assay, gp120 expression examination (by ELISA) and Western blotting. Results are shown in Figure 4. As clearly shown in Fig. 3, the production of gp120 tends to increase with the increasing HA titer of Sendai virus. Example 6 Analyses of SeVgp120 propagation and gp120 expression level in various types of cells

Using similar methods as those in Example 5 except for the use of various types of cells, HAU and gp120 expression levels (by ELISA) were assayed. Results are shown in Table 5.

Table 5

Cell type	Time (postinfection)	HAU	rgp120 (μg/ml)
CV-1	96	32	2.5
LLCMK2	48	16	0.5
CHO	55	4	0.46
NIH3T3	48	4	0.25
MT4	24	16	0.8
MOLT4	24	16	1.2

In the left-hand column of the Table are shown the postinfectional times of various types of cells transfected with SeVgp120. As a result, SeVgp120 propagation and gp120 expression were detected in all types of cells tested. Example 7. Studies on the expression of luciferase gene inserted into the Sendai viral vector in host cells.

In order to isolate the luciferase gene for inserting to vectors, the luciferase gene bounded by the engineered NotI sites on both termini was constructed by the standard PCR using a set of primers

[5'-AAGCGGCCGCCAAAGTTCACGATGGAAGAC-3') (30mer) (SEQ ID NO: 3)] and [5'-TGCGGCCGCGATGAACTTTCACCC-TAAGTTTTTCTTACTACGGATTATTACAATTTGGACTTTCCGCCC-3' (69mer) (SEQ ID NO: 4)

with "pHvluciRT4" as a template. The PCR product was cloned into the NotI window of pSeV18$^+$ to obtain Sendai virus vector to which the luciferase gene was inserted. Then, this recombinant vector was transfected into LLCMK2 cells, and inoculated into embryonated chicken eggs. Chorio-allantoic membranes of developing eggs were excised out, twice washed with cold PBS(-), and, after the addition of a lysis buffer (Picagene WAKO) (25 μl) and thorough mixing, centrifuged at 15,000 rpm for 2 min. To the supernatant (5 μl each) was added the substrate (IATRON) (50 μl), and the mixture was dispensed into each well of a 96-well plate. Fluorescent intensity was measured with a luminometer (Luminous CT-9000D, DIA-IATRON), and the enzyme activity was expressed as counts per second (CPS). As a result, an extremely high luciferase activity was detected with CV-1 cells at 24-h postinfection (Table 6). In this case, Sendai virus which did not carry the luciferase gene was used as control (represented by "SeV" in the table). Results obtained from two clones are shown in the table.

Table 6

Fluorescence intensity (counts/10 Sec)		
	Chorio-allantoic membrane	CV-1 (24h postinfection)
Luc/SeV	669187	
	2891560	8707815
SeV	69	48
	23	49

Industrial applicability

In the present invention, a system has been established allowing the efficient rescue of viral particles from cDNAs of negative strand viruses, and also a method has been developed enabling the production and amplification of "com-

plexes comprised of RNAs derived from disseminative specific negative strand RNA virus and viral structural components containing no nucleic acids so as to have the infectivity and autonomous RNA replicating capability but no disseminative potency". Since said complexes can replicate only within infected cells, these techniques are especially useful in the fields of gene therapy, etc. wherein therapeutical safety is highly appreciated.

Sequence Listing

SEQUENCE IDENTIFICATION NUMBER: 1

LENGTH: 38

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid (synthetic DNA)

SEQUENCE

TGCGGCCGCC GTACGGTGGC AATGAGTGAA GGAGAAGT 38

SEQUENCE IDENTIFICATION NUMBER: 2

LENGTH: 69

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid (synthetic DNA)

SEQUENCE

TTGCGGCCGC GATGAACTTT CACCCTAAGT TTTTVTTACT ACGGCGTACG TCATCTTTTT 60

TCTCTCTGC 69

SEQUENCE IDENTIFICATION NUMBER: 3

LENGTH: 30

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid (synthetic DNA)

SEQUENCE

AAGCGGCCGC CAAAGTTCAC GATGGAAGAC 30

SEQUENCE IDENTIFICATION NUMBER: 4

LENGTH: 69

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid (synthetic DNA)

SEQUENCE

TGCGGCCGCG ATGAACTTTC ACCTAAGTT TTTCTTACTA CGGATTATTA CAATTTGGAC 60

TTTCCGCCC 69

Claims

1. A complex comprising an RNA molecule derived from a specific disseminative negative strand RNA virus and viral structural components containing no nucleic acid, having the cell infectivity and capable of autonomously replicating RNA, but deficient in the disseminative capability.

2. The complex of Claim 1, wherein said specific RNA virus is a negative strand RNA having non-segmented genome.

3. The complex of Claim 1, wherein said specific RNA virus is Sendai virus.

4. An RNA molecule comprising Sendai viral RNA or Sendai viral cDNA, wherein said RNA molecule is defective in that at least more than one gene coding for M, F and HN proteins are deleted or inactivated.

5. A complex comprising the RNA molecule of Claim 4 and viral structural components derived from Sendai virus containing no nucleic acid, having the cell infectivity and capable of autonomously replicating RNA, but deficient in disseminative capability.

6. A DNA molecule comprising a template DNA capable of transcribing the RNA molecule of Claim 4 in vitro or in vivo.

7. The complex of any one of Claims 1-3 or 5, wherein the RNA molecule contained in said complex comprises a foreign gene.

8. The complex of Claims 3 or 5, wherein the RNA molecule contained in said complex comprises a foreign gene.

9. The RNA molecule of Claim 4 comprising a foreign gene.

10. The DNA molecule of Claim 6 comprising a foreign gene.

11. An inhibitor for RNA replication contained in the complex of any one of Claims 1-3, 5, 7 or 8 comprising an inhibitory

agent for the RNA-dependent RNA replication

12. A host whereto the complex of any one of Claims 1-3, 5, 7 or 8 can disseminate.

13. The host of Claim 12 comprising genes for the infectivity of the complex of any one of Claims 1-3, 5, 7 or 8 on its chromosomes, and capable of replicating the same copies of said complex when infected with it.

14. The host of Claims 12 or 13, wherein said host is animals, or cells, tissues, or eggs derived from it.

15. The host of Claim 14 wherein said animal is mammalian.

16. The host of Claim 14 wherein said animal is avian.

17. A host expressing genes for the infectivity of the complex of any one of Claims of 3, 5 or 8, and capable of replicating the same copies of said complex when infected with it.

18. A host comprising more than one gene of the M, F and HE genes derived from Sendai virus or genes having functions equivalent to them on its chromosomes.

19. A host comprising the M gene of Sendai virus or its functional equivalent genes on its chromosomes.

20. A host comprising the M, NP, P/C and L genes of Sendai virus on its chromosomes (wherein each gene may be substituted with its functional equivalent, respectively).

21. A host comprising the M, F and HN genes of Sendai virus on its chromosomes (wherein each gene may be substituted with its functional equivalent, respectively).

22. A host comprising the M, F, HN, NP, P/C and L genes on its chromosomes (wherein each gene may be substituted with its functional equivalent, respectively).

23. The host of any one of Claims 17-22, wherein said host is animal, or cell, tissue or egg derived from it.

24. The host of Claim 23, wherein said animal is mammalian.

25. The host of Claim 23, wherein said animal is avian.

26. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of Claims 1-3, 5, 7 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. enzymes required for replicating said RNA or said cRNA, or a unit capable of biosynthesizing said enzymes, and
c. proteins related to the infectivity of said complex, or a unit capable of biosynthesizing said proteins.

27. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of Claims 1-3, 5, 7 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. enzymes required for replicating said RNA or said cRNA, or a unit capable of biosynthesizing said enzymes, and
c. the host of any one of Claims 12-25.

28. A kit consisting of the following two components,

a. the complex of any one of Claims 1-3, 5, 7 or 8, and
b. the host of any one of Claims 12-25.

29. A kit consisting of the following three components,

a. the RNA molecule contained in any one of Claims 3, 5 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. all NP, P/C and L proteins of Sendai virus or a unit capable of biosynthesizing said proteins, and
c. proteins related to the infectivity of said complex, or a unit capable of biosynthesizing said proteins.

30. A kit consisting of the following three components,

a. the RNA molecule contained in the complex of any one of Claims 3, 5 or 8, or cRNA of said RNA, or a unit capable of biosynthesizing said RNA or said cRNA,
b. all NP, P/C and L proteins of Sendai virus, or a unit capable of biosynthesizing said proteins, and
c. the host of any one of Claims 17-25

31. A kit consisting of the following two components,

a. the complex of any one of Claims 3, 5 or 8, and
b. the host of any one of Claims 17-25.

32. A method for producing the complex of any one of Claims 1-3, 5, 7 or 8 by introducing all three components of Claims 26a, 26b and 26c into a host.

33. A method for producing the complex of any one of Claims 1-3, 5, 7 or 8 by introducing both components of Claims 27a and 27b in the host of Claim 27c.

34. A method for amplifying and producing the complex of Claim 28a by transfecting said complex to the host of Claim 28b.

35. A method for producing the complex of any one of Claims 3, 5 or 8 by introducing the three components of Claims 29a, 29b and 29c into a host.

36. A method for producing the complex of any one of Claims 3, 5 or 8 by introducing both components of Claims 30a and 30b into the host of Claim 30c.

37. A method for amplifying and producing the complex of Claim 31a by transfecting said complex into the host of Claim 31b.

38. The RNA molecule of Claim 9 wherein a gene corresponding to the M gene is deleted or inactivated.

39. The RNA molecule of Claim 9 wherein all the genes corresponding to the M, F and HN genes are deleted or inactivated.

40. A kit consisting of the following three components,

a. the RNA molecule of Claim 38,
b. the host of Claim 20, and
c. the host of Claim 19.

41. A method for preparing a complex by introducing the RNA molecule of Claim 40a into the host of Claim 40b, and amplifying and producing said complex by transfecting it to the host of Claim 40c.

42. A complex produced by the method of Claim 41.

43. A kit consisting of the following three components,

a. the RNA molecule of Claim 39,
b. the host of Claim 22, and
c. the host of Claim 21.

44. A method for amplifying and producing a complex by introducing the RNA molecule of Claim 43a into the host of

Claim 43b, and amplifying and producing said complex by transfecting it to the host of Claim 43c

45. A complex produced by the method of Claim 44.

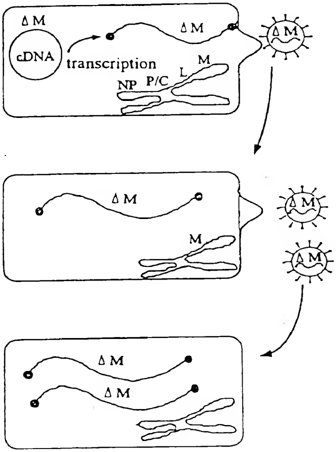
46. An inhibitor for RNA replication contained in the complex of Claims 42 or 45 comprising an inhibitory agent of the RNA-dependent RNA polymerase.

47. A method for preparing foreign proteins, wherein said method comprises processes of introducing the complex of Claim 7 to a host and recovering the expressed proteins.

48. The method for preparing foreign proteins of Claim 47, wherein the host is a cell expressing a group of genes, from among those related to the disseminative capability, which are deficient in the RNA molecule contained in the complex of Claim 7.

49. A culture medium or chorio-allantoic fluid containing the expressed foreign proteins obtained by inoculating the complex of Claim 7 to a host and recovering its culture medium or chorio-allantoic fluid.

Fig.1



A. Using a cell line expressing the NP,P/C and M proteins as the packaging cell,viral RNA is transcribed from cDNA deficient in the M gene, and one type of viral particles deficient in the M gene (ΔM type viral particles) is eventually produced.

B. These ΔM type viral particles can be packaged into the M gene expressing cells and recovered as particles.

C. Normal cells are infected with ΔM type viral particles, wherein the viral RNA is replicated within the cells, but viral particles are not formed.

Fig.2

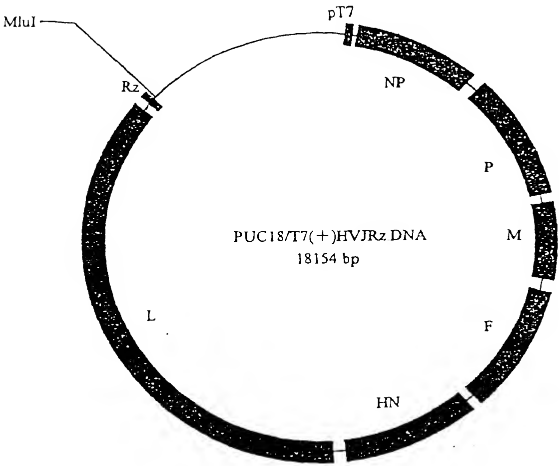


Fig.3

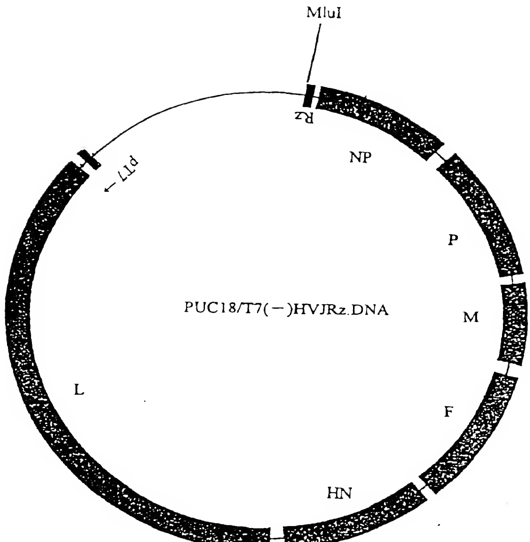
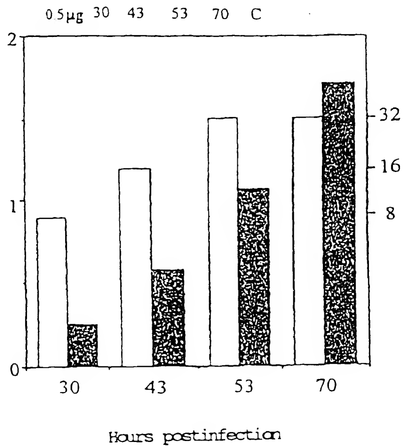


Fig.4


0.5µg 30 43 53 70 C
2
1
0
gp120(µ g/ml, ■)
32
16
8
HAU(□)
30
43
53
70
Hours postinfection

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/03063

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl⁶ C12N7/01, C12N15/45, C12N15/86, C12N5/10, C12N9/99, C12P21/02, A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl⁶ C12N7/01, C12N15/45, C12N15/86, C12N5/10, C12N9/99, C12P21/02, A61K48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSYS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Immunology, Vol. 126(3)(1981) Duane L. Peavy et al. "Inhibition of murine plaque-forming cell responses in vivo by rivavirin" p. 861-864	11, 46
X	Virology, Vol. 110(1981) Frank Malinoski et al. "Inhibitors of IMP Dehydrogenase Prevent Sindbis Virus Replication and Reduce GTP Levels in Aedes albopictus Cells" p. 281-291	11, 46
X	Journal of General Virology, Vol. 74(1993) Takemasa Sakaguti et al. "Expression of the HN, F, NP and M proteins of Sendai virus by recombinant vaccina viruses and their contribution to protective immunity against Sendai virus infections in mice" p. 479-484 (Refer to Fig. 1 LLCMK2 Cell)	12, 14-15
A	Journal of Virology, Vol. 67(8)(1993) Philippe Calain et al. "The Rule of Six, a Basic Feature for Efficient Replication of Sendai Virus	1 - 49

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
January 14, 1997 (14. 01. 97)	January 28, 1997 (28. 01. 97)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/03068

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Defective Interfering RNA" p. 4822-4830	
A	Journal of Virology, Vol. 68(12)(1994) W. Willenbrink et al. "Long-Term Replication of Sendai Virus Defective Interfering Particle Nucleocapsids in Stable Helper Cell Lines" p. 8413-8417	1 - 49
A	Annu. Rev. Microbiol., Vol. 47(1993) Adolfo Garcia-Saste et al. "Genetic Manipulation of Negative-Strand RNA Virus Genomes" p. 765-790	1 - 49
A	Journal of Virology, Vol. 66(12)(1992) K.H. Park et al. "In Vivo Model for Pseudo-Templated Transcription in Sendai Virus" p. 7033-7039	1 - 49
A	Cell, Vol. 59(1989) Willem Luytjes et al. "Amplification, Epression, and Packaging of a Foreign Gene by Influenza Virus" p. 1107-1113	1 - 49
A	JP, 4-211377, A (Schweiz Serum & Imp), August 3, 1992 (03. 08. 92) & EP, 440219, A & CA, 2035386, A & AU, 9170074, A	1 - 49

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